AD_____

Award Number: DAMD17-00-1-0558

TITLE: Targeting Angiogenic Factors Contributing to Etiology and Progression of Human Ovarian Cancer

PRINCIPAL INVESTIGATOR: Richard J. Pietras, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of California at Los Angeles
Los Angeles, California 90095-1406

REPORT DATE: September 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Annual (1 Sep 00 - 31 Aug 01)

4. TITLE AND SUBTITLE
Targeting Angiogenic Factors Contributing to Etiology and Progression of Human Ovarian Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0558

6. AUTHOR(S)
Richard J. Pietras, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California at Los Angeles
Los Angeles, California 90095-1406

E-Mail:

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Development of human ovarian cancer depends, in part, on formation of an adequate blood supply. Tumor angiogenesis is essential for cancer growth, and vascular endothelial growth factor (VEGF) is important in stimulating growth of vascular endothelial cells. VEGF is produced by many ovarian cancers, and our data show that VEGF secretion is markedly up-regulated in ovarian cancers with HER-2 gene overexpression. Herceptin, an antibody to HER-2 receptor, has direct antitumor effects, but the antireceptor antibody also elicits a significant reduction in VEGF secretion from ovarian cancer cells, and, thereby, also retards ovarian tumor-associated angiogenesis. More complete suppression of angiogenesis can be elicited by treatments that suppress blood vessel proliferation, such as squalamine, an angiostatic steroid recently approved by the FDA as an orphan drug candidate for the treatment of ovarian cancer. In studies with ovarian cancer cells *in vivo*, squalamine elicits antitumor activity by suppressing the angiogenic action of several vascular growth factors including VEGF. This ongoing work evaluates the efficacy of squalamine alone and combined with other antitumor therapies, including cisplatin and Herceptin, in suppressing the growth of ovarian cancers with and without HER-2 gene overexpression.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Ovarian Cancer, squalamine, VEGF, HER-2/neu, MAP kinase, carboplatin, endothelial cells	12
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

Ovarian cancer is the most deadly gynecologic malignancy. About 26,500 women are diagnosed with this cancer each year and have an overall 5-year survival rate of only 47% (1, 2). For most patients, surgery alone does not cure the cancer due to the spread of tumors beyond the confines of the ovary, and management in the clinic often requires use of toxic chemotherapy regimens. The progressive growth of ovarian cancer depends, in part, on the formation of an adequate blood supply, and tumor angiogenesis has been reported to have prognostic significance in epithelial ovarian cancer (3). Therapy directed toward the vasculature of solid tumors is now being pursued as an important new direction in cancer treatment, because avascular tumors exhibit limited growth (4,5) and tumor aggressiveness and metastatic potential commonly correlate with tumor vascularity (6).

Vascular endothelial growth factor (VEGF) is produced by most solid tumors and elicits a mitogenic effect on tumor-associated endothelial cells (7, 8). VEGF binding to receptor tyrosine kinases triggers activation of downstream signaling enzymes, including MAP kinase, which, in turn, regulate gene expression and specific endothelial cell responses including proliferation, migration, differentiation, and apoptosis (9, 10). Several studies suggest that VEGF plays an important role in progression of ovarian cancer (3, 11, 12), and the ability of VEGF to increase vascular permeability (7, 8, 13) may also promote formation of malignant ascites (14). Growth factor pathways, such as those dependent on the HER-2 receptor, appear to up-regulate VEGF production in some solid tumors (15). Since HER-2 receptor is overexpressed in a significant number of ovarian cancers (16, 17), it may also play a role in promoting further growth of ovarian malignancy by increasing VEGF-dependent tumor angiogenesis.

Squalamine, a natural sterol from tissues of the dogfish shark (18), has significant antiangiogenic and antitumor activity in laboratory models of brain, breast and lung cancer (19-22). Squalamine is a 7,24-dihydroxylated 24-sulfated cholestane steroid conjugated to spermidine at C-3. Squalamine blocks endothelial cell growth and has inhibitory activity in chick embryo chorioallantoic membrane and rabbit corneal micropocket assays (19, 20, 23). This antiangiogenic agent may have good potential for clinical application because it inhibits endothelial cell proliferation induced by a wide range of growth factors, including VEGF (19). This inhibition may result, in part, from its interaction with endothelial cell surface proton pumps, thereby altering intracellular pH and impeding signaling by growth factors (24, 25). When administered as a single agent in nude mice with lung cancer xenografts, squalamine has limited antitumor activity, but the antiangiogenic steroid significantly enhances the antitumor efficacy of cisplatin and carboplatin/paclitaxel chemotherapies (20-22). Since platinum-based treatments are often used for human ovarian cancers (1, 2), squalamine in combination with cisplatin was studied to assess its utility as part of a coordinated attack against human ovarian cancers and their blood supply. One additional feature we were particularly interested in was the consequence of HER-2 oncogene overexpression for squalamine modulation of growth in ovarian tumor xenografts. Amplification and/or overexpression of HER-2 proto-oncogene in human cancers, including ovarian cancers, is often associated with poor clinical outcome (16,17), and human ovarian tumor cells with overexpression of HER-2 membrane receptor also exhibit resistance to cisplatin (26). We therefore examined whether the level of HER-2 expression in paired HER-2-transfected and non-transfected ovarian cancers influenced the degree of tumor growth inhibition seen with squalamine with or without concomitant platinum-based treatment.

BODY: RESEARCH PROGRESS

AIM 1) Evaluation of the angiogenic activity due to HER-2 gene overexpression in human ovarian cells.

1.a. *Squalamine Does not Affect VEGF Secretion in vitro for Ovarian Cancer Cells with or without HER-2 Gene Overexpression.*

HER-2 overexpression is generally thought to lead to tumor development through its effects on promoting uncontrolled cancer cell growth. However, recent findings suggest that HER-2 may also regulate cell survival

functions such as angiogenesis by promoting tumor production of VEGF (15). To explore how HER-2 may contribute to angiogenesis in ovarian cancer, we evaluated HER-2 effects on *in vitro* VEGF secretion by human ovarian tumor cells. Parent and HER-2-overexpressing ovarian 2008 cells were incubated for 72 h *in vitro*, and secretion of VEGF into conditioned media was measured by use of established enzyme-linked immunosorbant assay (ELISA) (13, 15) (Fig. 1). Parent ovarian cancer cells show significant secretion of VEGF, and, after transfection of ovarian cells with HER-2 gene to high levels, a further increase in VEGF secretion was found. In parallel *in vitro* studies, treatment of ovarian cancer cells with squalamine elicited no significant effect on secretion of VEGF (Fig. 1). Thus, HER-2 overexpression may contribute to angiogenesis through up-regulation of VEGF secretion in ovarian cancer, but squalamine is not antiangiogenic at this step in tumor-associated angiogenesis since it does not appear to directly affect secretion of VEGF by ovarian epithelial tumor cells.

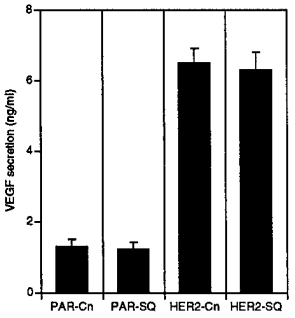

Fig. 1. VEGF secretion by ovarian cancer cells with or without HER-2 gene overexpression. Ovarian 2008 parental cells (PAR) (27) and ovarian 2008 HER-2-overexpressing cells (HER2) (26,28, 29) were grown *in vitro*, either with control media (Cn) or media with squalamine (SQ;16 μM). After 72 hrs, media were collected and processed for ELISA assay of VEGF (13,30). Data represent mean ± SEM units of VEGF secretion.

AIM 2) Assessment of the biologic activities of squalamine, a newly-synthesized antiangiogenic steroid, using human vascular endothelial cells *in vitro*.

2.a. *Squalamine Blocks VEGF-Stimulated Proliferation of Endothelial Cells in vitro.*

To assess potential biologic mechanisms for antiangiogenic and antitumor effects of squalamine noted previously, human umbilical vein endothelial cells (HUVEC) were grown *in vitro*. VEGF elicits significant proliferation of HUVEC cells by 72 h. In the absence of VEGF, squalamine has no effect on proliferation or survival of HUVEC cells. However, in the presence of VEGF, squalamine elicited a significant reduction in VEGF-induced endothelial cell proliferation ($P<0.001$) (Fig. 2). This growth-suppressive effect of squalamine appears restricted to endothelial cells since the compound had no direct inhibitory effect on the proliferation of ovarian 2008 cancer cells, either with or without HER-2 gene overexpression (Fig. 2).

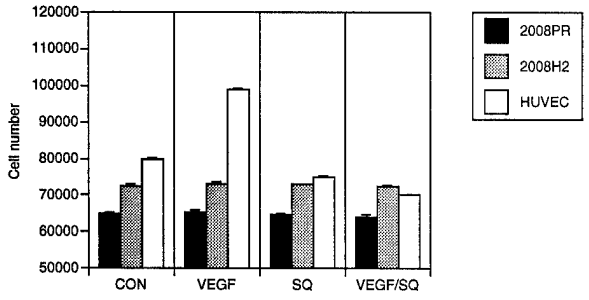

Fig. 2. Squalamine inhibits VEGF-induced proliferation of endothelial cells *in vitro*, but does not directly affect the growth of ovarian epithelial tumor cells *in vitro*. Human umbilical vein endothelial cells (HUVEC), ovarian 2008 parental cells (2008PR) and ovarian 2008 HER-2-overexpressing cells (2008H2) were grown *in vitro* in the presence of vascular endothelial cell growth factor (VEGF; 20 ng/ml) or control vehicle (CON), squalamine (SQ; 16 μM) or combinations of VEGF with squalamine (VEGF/SQ) for three days. In additional control studies, squalamine at 8 μM and 16 μM also elicited no direct effect on proliferation *in vitro* of CAOV3 ovarian cancer cells with HER-2 overexpression (data not shown). All data are from triplicate determinations of cell numbers, with results presented as mean ± SEM.

2.b. *Squalamine Blocks VEGF-Induced Activation of MAP Kinase in vitro.*

VEGF exerts its biologic effects by binding with receptor tyrosine kinases, notably Flt-1 and Flk-1/KDR, present at the surface of endothelial cells (9). Post-receptor signal transduction regulates the effects of VEGF, and the proliferative action of VEGF in endothelial cells has been associated with VEGF-induced tyrosine phosphorylation and stimulation of mitogen-activated protein kinases (MAP kinase), extracellular signal-regulated kinase ERK-1 (p44 ^{MAPK}) and ERK-2 (p42 ^{MAPK}) (9, 10). On the assumption that blockade of endothelial cell proliferation by squalamine may occur, in part, by suppression of MAP kinase signaling cascades induced by growth factors, VEGF-induced tyrosine phosphorylation of MAP kinases was assessed. As expected, VEGF promotes tyrosine phosphorylation of MAP kinase isoforms, with maximal effects evident by 10 min (Fig. 3). However, after administration of squalamine, the VEGF-stimulated phosphorylation of MAP kinase isoforms is significantly suppressed, especially after 30 min exposure to VEGF (Fig. 3).

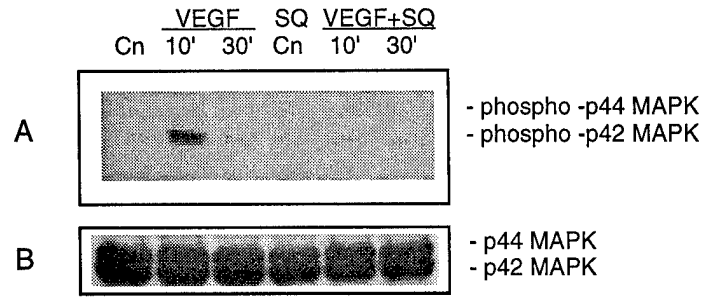

Fig. 3. Squalamine blocks VEGF-stimulated tyrosine phosphorylation of MAP kinase in HUVEC cells *in vitro*. Quiescent HUVEC cells were treated with control vehicle (Cn), VEGF (50 ng/ml), squalamine (SQ ; 1.6 μM) or VEGF in combination with squalamine (VEGF + SQ) for 10 or 30 minutes *in vitro*. Lysates were prepared and processed as before (28,31). A, Western blotting was performed with anti-phospho-p44/42 MAP kinase monoclonal antibody as previously (32). B, Paired samples were used for Western blotting with anti-p44/42 MAP kinase monoclonal antibody to confirm similar total protein load in each lane. Treatment of HUVEC cells *in vitro* with squalamine at 0.8 μM elicited a similar block of VEGF-stimulated tyrosine phosphorylation of MAP kinase (data not shown).

AIM 3) Investigation of the efficacy of squalamine alone and combined with other antitumor agents in blocking the *in vivo* growth and progression of human ovarian cancer xenografts in nude mice.

3.a. *Squalamine and Platinum-Based Chemotherapeutic Agents Block Growth of Ovarian Tumor Xenografts in vivo.*

Potential antitumor effects of the angiostatic steroid squalamine were assessed in murine tumor xenografts in the absence and presence of cisplatin chemotherapy. Human ovarian 2008 cancer cells without (Fig. 4A) or with HER-2 overexpression (Fig. 4B) were grown as subcutaneous tumors in nude mice. Tumors were grown to 150-200 mm^3 in size. Then, animals with established tumors were treated with control solution, cisplatin alone (4 mg/kg) on day 1, squalamine alone (2 mg/kg) on days 1-10, or cisplatin (day 1) in combination with squalamine (days 1-10). Doses of cisplatin that resulted in only partial growth inhibition (26,29) were chosen for these combination experiments in order to ensure use of the chemotherapeutic agant at a level that would not totally suppress tumor growth, thus allowing detection of any potential additive effects of a squalamine-cisplatin interaction. By 28 days, both 2008 parental and HER-2-overexpressing tumors showed little overall response to therapy with cisplatin alone although there was some early indication of tumor response to cisplatin treatment. Squalamine elicited a partial reduction in tumor size as compared to controls ($P<0.01$) in both 2008 parental (Fig. 4A) and HER-2-overexpressing (Fig. 4B) tumors. More profound tumor growth inhibition (94-

95% of controls) was elicited by combined treatment with squalamine and cisplatin (P<0.001) in both 2008 parental (Fig. 4A) and HER-2-overexpressing (Fig. 4B) cancers.

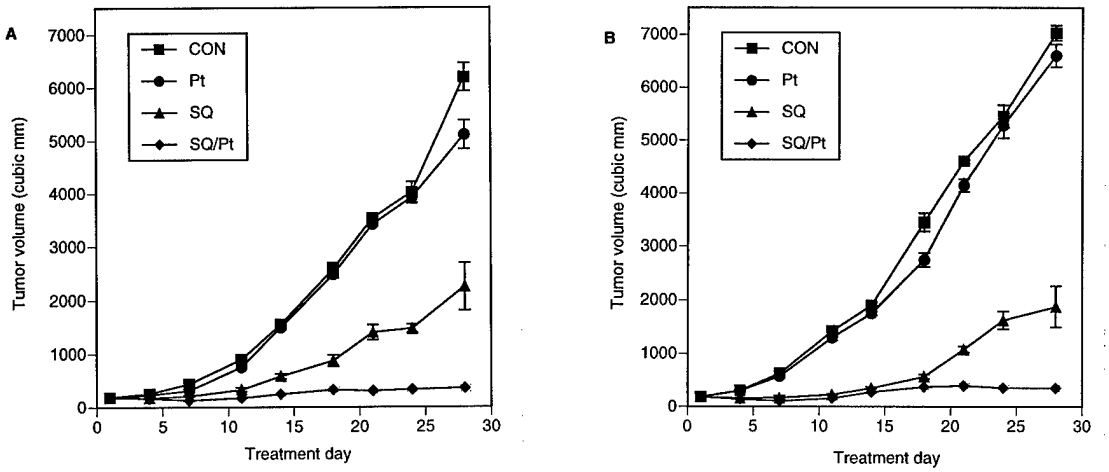

Fig. 4. Squalamine inhibits growth of ovarian 2008 parental and HER-2-overexpressing tumor xenografts in nude mice and enhances the cytotoxic effects of cisplatin. A, Ovarian 2008 parental tumor cells were inoculated subcutaneously in nude mice as before (26,29). B, Ovarian 2008 HER-2-overexpressing cancer cells were inoculated subcutaneously in nude mice (26,29). After 7 days, animals with tumors of comparable size were randomized to treatment with control vehicle (CON), squalamine (SQ; 2 mg/kg on days 1-10), cisplatin (Pt; 4 mg/kg on day 1), or cisplatin administered in combination with squalamine (SQ/Pt). Results are expressed as mean ± SEM for tumor volumes (mm^3) measured over a 28-day experimental period. Based on previous work (21,26,29,35), cisplatin (4mg/kg) was administered at doses less than the MTD in an attempt to allow any additive effects of combination treatment with cisplatin and squalamine to be detected (21,26). The 2mg/kg squalamine dose was selected as being the minimal daily dose previously shown to be significantly active in a tumor xenograft model in combination with a platinum agent (22). All animal studies were conducted according to protocols approved by the UCLA animal research committee.

The antitumor effects of squalamine with and without platinum-based chemotherapy were also assessed using a different ovarian tumor xenograft, CAOV3, that has been transfected to exhibit HER-2 overexpression (Fig. 5A). After tumor growth to 50-60 mm^3, animals were treated with control solution, carboplatin alone (60 mg/kg) on day 1, squalamine alone (2 mg/kg) on days 1-10, or carboplatin (day 1) in combination with squalamine (days 1-10). By 28 days, CAOV3 HER-2-overexpressing tumors showed minimal response to therapy with carboplatin alone (Fig. 5A). As with the 2008 tumors, squalamine as a single agent elicited a partial reduction in CAOV3 tumor size as compared to controls (P<0.05) (Fig. 5A). More marked inhibition of tumor growth was elicited by combined treatment with squalamine and carboplatin (P<0.001; Fig. 5A).

The tumor growth inhibition seen with combined squalamine and platinum-based chemotherapeutics for both human ovarian tumor lines persisted for up to 18 days following cessation of squalamine treatment. We therefore investigated how long bioactivity persisted with combined cisplatin and squalamine treatment of HER-2-overexpressing CAOV3 tumors by maintaining the dual therapy animal cohort until the mean tumor size for these animals reached 500 mm^3 (Fig. 5B). After tumor growth to 50-60 mm^3, animals with established tumors were treated with control solution, cisplatin alone (4 mg/kg) on day 1, squalamine alone (2 mg/kg) on days 1-10, or cisplatin (day 1) combined with squalamine (days 1-10). As compared with control tumor xenografts, the calculated tumor growth delay in established tumors was 7 days for cisplatin therapy alone, 28 days for squalamine treatment alone, and 91 days for squalamine with cisplatin (Fig. 5B). Combined squalamine-cisplatin therapy was nontoxic as assayed by no animal death or significant weight loss during the study period.

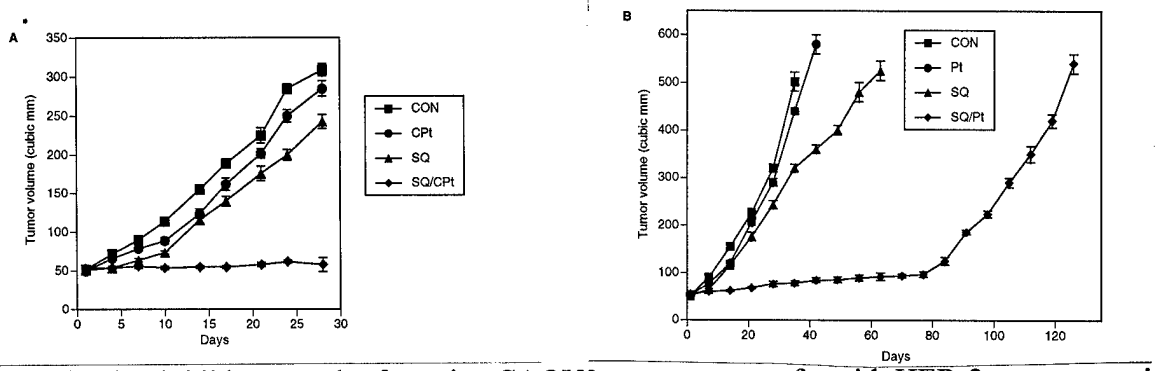

Fig. 5. Squalamine inhibits growth of ovarian CAOV3 tumor xenografts with HER-2 overexpression and enhances cytotoxic effects of cisplatin and carboplatin. A, CAOV3 ovarian cancer cells were inoculated SC in nude mice. After 7 days, animals with tumors of comparable size were randomized to treatment with control solution (CON), squalamine (SQ; 2 mg/kg on days 1-10), carboplatin (CPt; 60 mg/kg on day 1), or carboplatin given in combination with squalamine (SQ/CPt). Results are given as mean ± SEM for tumor volumes (mm^3) measured over a 28-day period. B, CAOV3 ovarian cancer cells were inoculated SC in nude mice. After 7 days, animals with tumors of comparable size were randomized to treatment with control solution (CON), squalamine (SQ; 2 mg/kg on days 1-10), cisplatin (Pt; 4 mg/kg on day 1), or cisplatin with squalamine (SQ/Pt). Tumor volumes (mm^3) are expressed as mean ± SEM for measurements to assess tumor growth delay due to treatments (20). Based on previous work (21,26,29,33), cisplatin (4mg/kg) and carboplatin (60 mg/kg) were given at doses < MTD to allow additive effects of combined treatment with platinum agents and squalamine to be detected (21,26). Tumor growth delay was calculated by graphing the volume of each treatment group and calculating the number of additional days it took to reach 500 mm^3 compared with control (20).

3.b. *Squalamine and Cisplatin Promote Ovarian Tumor Cell Apoptosis in vivo.*

To assess molecular effects of squalamine and cisplatin, ovarian 2008 parent and HER-2-overexpressing tumor xenografts remaining after treatments with squalamine, cisplatin or a combination of the reagents were harvested and assessed for ovarian tumor cell apoptosis *in vivo*. For evaluation of apoptosis, the modified TUNEL assay (34, 35) was performed on tissue sections. The assays showed evidence of increased apoptosis in ovarian 2008 parental tumor cells treated with squalamine alone, cisplatin or combined cisplatin-squalamine as compared to appropriate controls (all at $P<0.05$) (Fig. 6A). The 2008 HER-2-overexpressing ovarian tumors displayed less apoptotic activity than 2008 parental cancers with all treatments ($P<0.05$). Although apoptosis tended to be higher after administration of either squalamine or cisplatin alone, only treatment with squalamine in combination with cisplatin elicited a significant increase in the extent of apoptosis of HER-2-overexpressing ovarian cancers ($P<0.001$) (Fig. 6A). The results suggest that squalamine enhances cytotoxic effects of cisplatin chemotherapy for human ovarian cancer cells by increasing levels of tumor cell apoptosis produced by cisplatin exposure, either with or without HER-2 oncogene overexpression.

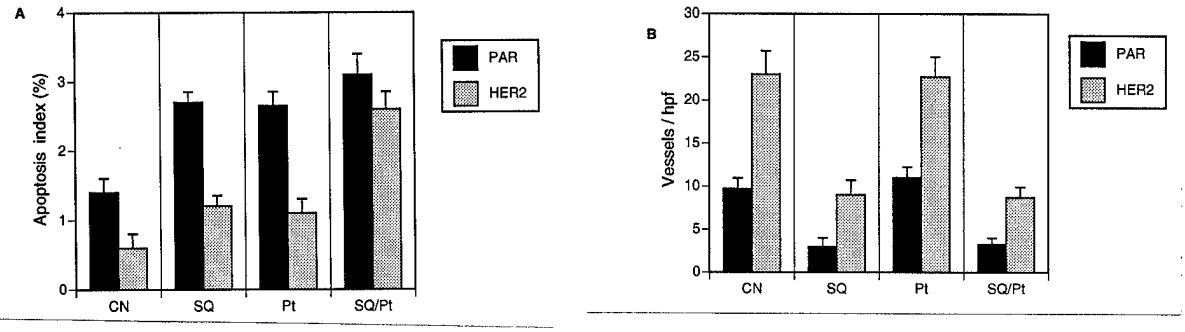

Fig. 6. Effects of squalamine and cisplatin on ovarian tumor cell apoptosis and tumor-associated angiogenesis *in vivo*. At the end of experiments, tumors were excised, fixed and embedded using established procedures (36). A, A modified TUNEL assay was used in ovarian 2008 parental (PAR) and HER-2-overexpressing (HER2)

tumor sections to compare tumor cell apoptosis *in vivo* among the different treatment groups, including control (CN), squalamine (SQ; 2 mg/kg on days 1-10), cisplatin (Pt; 4 mg/kg on day 1), and squalamine-cisplatin (SQ/Pt; squalamine combined with cisplatin) (Fig. 4). The apoptotic index was assessed as before (23,34,35, 37). B, Squalamine and cisplatin inhibit tumor-associated angiogenesis in ovarian 2008 parental and HER-2-overexpressing tumor xenografts in nude mice. Immunohistochemical staining for vWF was used to quantitate blood vessel density (blood vessels /hpf) in tumor sections as before (23,34). The treatment groups included control (CN), squalamine (SQ; 2 mg/kg on days 1-10), cisplatin (Pt; 4 mg/kg on day 1), squalamine-cisplatin (SQ/Pt; squalamine plus cisplatin) (Fig. 4A,B). Results are expressed as mean ± SEM of appropriate values.

3.c. *Squalamine Down-Regulates Ovarian Tumor-Associated Angiogenesis but not VEGF Production in vivo.*

Tissue sections of parent and HER-2-overexpressing 2008 tumor xenografts remaining after treatments with squalamine, cisplatin or a combination of the reagents (Fig. 4) were prepared for immunohistochemical staining with human von Willebrand Factor (vWF) to detect blood vessels (34). On scoring of tumor microvessel density, 2008 HER-2-overexpressing tumors exhibited more angiogenic activity than 2008 parental cancers ($P<0.001$) (Fig. 6B). Treatment with squalamine alone elicited a reduction of tumor-associated blood vessel density for either ovarian tumor ($P<0.001$) (Fig. 6B), and the immunohistochemical analyses also revealed a reduction of tumor-associated angiogenesis in mice treated with cisplatin plus squalamine ($P<0.01$) (Fig. 6B). No significant differences in microvessel density were found between groups treated with cisplatin alone and controls. The results suggest that squalamine is antiangiogenic for ovarian cancer cells with or without HER-2 overexpression. Squalamine-induced suppression of tumor microvessels is also a sustainable event since it was noted up to 18 days following the last squalamine dose.

KEY RESEARCH ACCOMPLISHMENTS

- Profound growth inhibition was elicited by squalamine alone and by combined treatment with squalamine and cisplatin for both parental and HER-2-overexpressing ovarian tumor xenografts.
- Immunohistochemical evaluation of tumors revealed decreased microvessel density and increased apoptosis. Although HER-2-overexpressing tumors had more angiogenic and less apoptotic activity than parental cancers, growth of both tumor types was similarly suppressed by treatment with squalamine combined with cisplatin.
- In *in vitro* studies, we found that squalamine does not directly affect proliferation of ovarian cells. However, squalamine significantly blocked VEGF-induced activation of MAP kinase and cell proliferation in human vascular endothelial cells.
- The results suggest that squalamine is antiangiogenic for ovarian cancer xenografts and appears to enhance cytotoxic effects of cisplatin chemotherapy independent of HER-2 tumor status.

REPORTABLE OUTCOMES

Presentations

1. "Squalamine and Cisplatin Block Angiogenesis and Growth of Human Ovarian Cancer Cells With or Without HER-2 Gene Overexpression". Presented at Jonsson Comprehensive Cancer Center Seminar Series, UCLA (2001).

No publications, patents, development of cell lines, informatics or additional funding or research opportunities to be reported at this time.

CONCLUSIONS

The potential role of squalamine, a natural antiangiogenic sterol, in treatment of ovarian cancers with or without standard cisplatin chemotherapy was assessed. Since HER-2 gene overexpression is associated with cisplatin resistance *in vitro* and promotion of tumor angiogenesis *in vivo*, the response of ovarian cancer cells with or without HER-2 gene overexpression to squalamine and cisplatin was also evaluated in tumor xenograft models and in tissue culture. Profound growth inhibition was elicited by squalamine alone and by combined treatment with squalamine and cisplatin for both parental and HER-2-overexpressing ovarian tumor xenografts. Immunohistochemical evaluation of tumors showed decreased microvessel density and increased apoptosis. Although HER-2-overexpressing tumors had more angiogenic and less apoptotic activity than parental cancers, growth of both tumor types was similarly suppressed by treatment with squalamine combined with cisplatin. In *in vitro* studies, we found that squalamine does not directly affect proliferation of ovarian cells. However, squalamine significantly blocked VEGF-induced activation of MAP kinase and cell proliferation in human vascular endothelial cells. The results suggest that squalamine is antiangiogenic for ovarian cancer xenografts and appears to enhance cytotoxic effects of cisplatin chemotherapy independent of HER-2 tumor status. Further experiments are ongoing in accord with our statement of work

REFERENCES

1. Ozols, R. F. Paclitaxel plus carboplatin in the treatment of ovarian cancer. Sem. Oncol., *26*: 84-89, 1999.
2. Stiff, P. J. The challenge of treating advanced ovarian cancer. Cancer Mgmt., *2*: 1-5, 1997.
3. Alvarez, A. A., Krigman, H. R., Whitaker, R. S., Dodge, R. K., and Rodriguez, G. C. The prognostic significance of angiogenesis in epithelial ovarian carcinoma. Clin. Cancer Res., *5*: 587-591, 1999.
4. Folkman, J. Tumor angiogenesis: therapeutic implications. New Engl. J. Med., *285*: 1182-1186, 1971.
5. Gimbrone, M. A. Jr., Leapman, S. B., Cotran, R. S., and Folkman, J. Tumor dormancy in vivo by prevention of neovascularization. J. Exp. Med., *136*: 261-276, 1972.
6. Teicher, B. A. Angiogenesis and cancer metastases: therapeutic approaches. Crit. Rev. Oncology Hematology, *20*: 9-39, 1995.
7. Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., and Connolly, D. T. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science, *240*: 1309-1312, 1989.
8. Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V., and Ferrara, N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science, *246*: 1306-1309, 1989.
9. Soker, S., Fidder, H., Neufeld, G., and Klagsbrun, M. Characterization of novel vascular endothelial growth factor (VEGF) receptors on tumor cells that bind VEGF165 via its exon 7-encoded domain. J. Biol. Chem., *271*: 5761-5767, 1996.
10. Rousseau, S., Houle, F., Landry, J., and Huot, J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. Oncogene, *15*: 2169-2177, 1997.
11. Paley, P. J., Staskus, K. A., Gebhard, K., Mohanraj, D., Twiggs, L. B., Carson, L. F., and Ramakrishnan, S. Vascular endothelial growth factor expression in early stage ovarian carcinoma. Cancer, *80*: 98-106, 1997.
12. Yamamoto, S., Konishi, I., Mandai, M., Kuroda, H., Komatsu, T., Nanbu, K., Sakahara, H., and Mori, T. Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels. Brit. J. Cancer, *76*: 1221-1227, 1997.
13. Ferrara, N., Winer, J., Burton, T., Rowland, A., Siegel, M., Phillips, H. S., Terrell, T., Keller, G. A., and Levinson, A. D. Expression of vascular endothelial growth factor does not promote transformation but confers a growth advantage in vivo to Chinese Hamster Ovary cells. J. Clin. Invest., *91*: 160-170, 1993.
14. Zebrowski, B. K., Liu, W., Ramirez, K., Akagi, Y., Mills, G. B., and Ellis, L. M. Markedly elevated levels of vascular endothelial growth factor in malignant ascites. Ann. Surg. Oncol., *6*: 373-378, 1999.

15. Petit, A. M., Rak, J., Hung, M. C., Rockwell, P., Goldstein, N., Fendly, B., and Kerbel, R. S. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. Am. J. Pathol., *151*: 1523-1530, 1997.
16. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science, *244*: 707-712, 1989.
17. Hellstrom, I., Goodman, G., Pullman, J., Yang, Y. and Hellstrom, K.E. Overexpression of HER-2 in ovarian carcinomas. Cancer Res., 61 : 2420-2423, 2001.
18. Moore, K. S., Wehrli, S., Roder, H., Rogers, M., Forrest, J. N. Jr., McCrimmon, D., and Zasloff, M. Squalamine: an aminosterol antibiotic from the shark. Proc. Natl. Acad. Sci. USA, *90*: 1354-1358, 1993.
19. Sills, A. K. Jr., Williams, J. I., Tyler, B. M., Epstein, D. S., Sipos, E. P., Davis, J. D., McLane, M. P., Pitchford, S., Cheshire, K., Gannon, F. H., Kinney, W. A., Chao, T. L., Donowitz, M., Laterra, J., Zasloff, M., and Brem, H. Squalamine inhibits angiogenesis and solid tumor growth in vivo and perturbs embryonic vasculature. Cancer Res., *58*: 2784-2792, 1998.
20. Teicher, B. A., Williams, J. I., Takeuchi, H., Ara, G., Herbst, R. S., and Buxton, D. Potential of the aminosterol, squalamine in combination therapy in the rat 13,762 mammary carcinoma and the murine Lewis lung carcinoma. Anticancer Res., *18*: 2567-2573, 1998.
21. Schiller, J. H., and Bittner, G. Potentiation of platinum antitumor effects in human lung tumor xenografts by the angiogenesis inhibitor squalamine: effects on tumor neovascularization. Clin. Cancer Res., *5*: 4287-4294, 1999.
22. Williams, J.I., Weitman, S., Gonzalez, C., Jundt, C., Marty, J., Stringer, S., Hunt, J., Holroyd, K., McLane, M., Zasloff, M. and von Hoff, D.D. Squalamine treatment of chemoresistant human tumors in female nu/nu mice enhances platinum-based chemotherapies. Clin. Cancer Res., 7: 724-733 (2001).
23. Williams, J. I. Squalamine-a new angiostatic steroid. in *Antiangiogenic agents* (ed. Teicher, B. A.) Totowa, NJ: Humana Press, 1999.
24. Akhter, S., Nath, S. K., Tse, C. M., Williams, J., Zasloff, M., and Donowitz, M. Squalamine, a novel cationic steroid, specifically inhibits the brush-border Na+/H+ exchanger isoform NHE3. Am. J. Physiol., *276*: C136-C144, 1999.
25. Eckhardt, S. G. Angiogenesis inhibitors as cancer therapy. Hospital Pract., *1*: 63-78, 1999.
26. Pegram, M. D., Finn, R. S., Arzoo, K., Beryt, M., Pietras, R. J., and Slamon, D. J. The effect of HER-2/neu overexpression on chemotherapeutic drug sensitivity in human breast and ovarian cancer cells. Oncogene, *15*: 537-547, 1997.
27. Disaia, P. J., Sinkovics, J., Rulege, F. N., and Smith, J. P. Cell-mediated immunity to human malignant cells. A brief review and further studies with two gynecologic tumors. Am. J. Obstet. Gynecol., *114*: 979-989, 1972.
28. Chazin, V., Kaleko, M., Miller, A., and Slamon, D. J. Transformation mediated by the human HER-2 gene independent of the epidermal growth factor receptor. Oncogene, *7*: 1859-1865, 1992.
29. Pietras, R. J., Fendly, B. M., Chazin, V. R., Pegram, M. D., Howell, S. B., and Slamon, D. J. Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. Oncogene, *9*: 1829-1838, 1994.
30. Goldman, C. K., Kim, J., Wong, W. L., King, V., Brock, T., and Gillespie, G. Y. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells. Mol. Biol. Cell, *4*: 121-133, 1993.
31. Migliaccio, A., Castoria, G., De Falco, A., Di Domenico, M., Galdiero, M., Nola, E., Chambon, P., and Auricchio, F. In vitro phosphorylation and hormone binding activation of the synthetic wild type human estradiol receptor. J. Steroid Biochem. Mol. Biol., *38*: 407-413, 1991
32. Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Tecle, H., Barrett, S., Bridges, A., Przybranowski, S., Leopold, W. R., and Saltiel, A. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nature Medicine, *5*: 810-816, 1999.

33. Plumb, J.A., Strathdee, G., Sludden, J., Kaye, S.B., and Brown, R. Reversal of drug resistance in human tumor xenografts by 2'-deoxy-5-azacytidine-induced demethylation of the hMLH1 gene promoter. Cancer Res., 60: 6039-6044, 2000.
34. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: An endogenous inhibitor of angiogenesis and tumor growth. Cell, *88*: 277-285, 1997.
35. Ellis, R. E., Yuan, J. Y., and Horvitz, H. R. Mechanisms and Functions of Cell Death. Annu. Rev. Cell. Biol., *7*: 663-698, 1991.
36. Luna, L. G. Manual of Histologic Staining: Methods of the Armed Forces Institute of Pathology. New York, NY: McGraw-Hill, 1968.
37. Steller, H. Mechanisms and Genes of Cellular Suicide. Science, *267*: 1445-1449, 1995.